FORSKOLIN, AN INDUCER OF CAMP, UP-REGULATES ACETYLCHOLINESTERASE EXPRESSION AND PROTECTS AGAINST ORGANOPHOSPHATE EXPOSURE IN NEURO2A CELLS

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ABSTRACT

Bioscavenger prophylactic therapy using purified human acetylcholinesterase (AChE) or butylcholinesterase (BChE) is a promising treatment for future protection against chemical warfare agent (CWA) exposure. However, the potential limitations of this approach has led to our investigation of alternative bioscavenger approaches using Forskolin, an inducer of cyclic AMP (cAMP). The optimal dose found for repeated treatment of Forskolin was 12-24 μM. Exposure to the organophosphate diisopropylflurophophate (DFP) showed that Forskolin treatment protects the cells from DFP cytotoxicity. These results indicate that transcriptional inducers, such as Forskolin, can sufficiently up-regulate AChE production and protect cells against CWA exposure.

INTRODUCTION

Exposure to organophosphorous (OPs) chemical warfare nerve agent result in several biological effects; mainly, the inhibition of acetylcholinesterase (AChE) and decreased catalyzed hydrolysis of the enzyme, causing excessive accumulation of extracellular acetylcholine (ACh). The presence of excess ACh in the synaptic cleft triggers hyper-activation of ACh receptors and leads to various toxic effects, including hyper-secretions, convulsions, respiratory distress, coma, and death. Acute treatment of OP poisoning consists of combined administration of an AChE re-activator (an oxime), a muscarinic ACh receptor antagonist (e.g. atropine), and an anticonvulsant (e.g. diazepam) [1;2]. Recent investigations show that prophylactic enzyme therapy using purified fetal bovine AChE or human plasma butyrylcholinestrase (BChE) appears to be a more promising treatment against OP exposure [3;3;4]. However, the complex structure of the cholinesterases, posttranslational modifications and genetic variations, which could lead to antibody generation, raise certain concerns about this approach. Intense labor, the large amounts of serum needed for purification, low yield of purified enzyme and the high-dose regimens required for therapy are additional drawbacks that have necessitated a search for alternative approaches to nerve agent bio-scavenger treatment. One new approach is to induce the expression of endogenous cholinesterases using transcriptional inducers. In addition to complementing the studies on the regulation of cholinesterase expression, transcriptional upregulation of cholinesterases is also useful to produce large quantities of recombinant enzymes to facilitate purification of cholinesterases.

Accumulating evidence suggests that the cellular expression of AChE is regulated both transcriptionally and posttranscriptionally. The promoter of human AChE is activated by a cyclic AMP (cAMP)-dependent pathway and augmented by cAMP [5;6]. Forskolin, a strong inducer of intracellular cAMP, also has been shown to induce AChE expression. Several binding sites of vitamin D3 and 17β-estradiol have been reported in human AChE promoters [7] and both these factors enhance transcription of the AChE gene. Constitutively activated $G\alpha_s$ and activation transcription factor-1 showed 10- and 4-fold increase in AChE activity. The RNA-binding protein HuD upregulates AChE posttranslationally by binding to the 3′

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
16 NOV 2004	**N/A**	**-**

4. TITLE AND SUBTITLE
Forskolin, An Inducer Of Camp, Up-Regulates Acetylcholinesterase Expression And Protects Against Organophosphate Exposure In Neuro2a Cells

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Biochemical Pharmacology, Division of Biochemistry, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001849, 2004 Scientific Conference on Chemical and Biological Defense Research. Held in Hunt Valley, Maryland on 15-17 November 2004.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**11**	

untranslated region. Agonists of P2Y(1) receptors and peroxisome proliferator-activated receptor gamma strongly induce the expression of AChE in neuroblastoma cells [8-10]. Neuronal-glial interactions mediated by interleukin-1 also significantly increase the expression of AChE [11]. This evidence suggests that the constitutive expression of AChE can be significantly increased by transcriptional inducers.

We investigated the effects of the transcriptional enhancer, Forskolin, on the upregulation of endogenous cholinesterase expression in neuronal cell lines to detoxify OPs. Forskolin induces gene expression by enhancing the binding of transcription factors through inhibition of histone deacetylase, which leads to increased histone acetylation and decondensing of the chromatin [12;13]. We demonstrate that Forskolin significantly enhances the expression of both intracellular and extracellular AChE in the mouse neuroblastoma cell line, Neuro 2A. Furthermore, the increased level of cholinesterases in Forskolin-treated cells allow bio-scavenging of the OPs and significantly protect the cells from organophosphate diisopropylfluorophosphate (DFP) toxicity. These results indicate that transcriptional inducers of cholinesterases are potential new ways to treat nerve agent exposure.

MATERIALS AND METHODS

NEURONAL CELL LINES AND CELL CULTURE: Mouse neuroblastoma cell line Neuro 2A was obtained from American Type Culture Collection (Manassas, VA). Neuro 2A cell lines were cultured in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The cells were cultured at 37° C in an incubator with humidified air and 5% CO_2.

DEPLETION OF CHOLINESTERASES FROM FETAL BOVINE SERUM: To minimize the interference during transcriptional induction and enzyme assay, serum cholinesterases were depleted using affinity chromatography using procainamide immobilized gel that inhibits serine proteases. In brief, 10 ml procainamide gel was packed in a glass column and washed with 0.1 M phosphate buffer, pH 8.0. Fetal bovine serum (100 ml) was loaded on to the column. The first 20 ml of the flow-through was discarded, since it could be diluted with the phosphate buffer. The remaining 80 ml of the flow-through was collected and sterilized by passing through 0.22-0.45 micron filters. Depletion of the serum AChE was confirmed by comparing the enzyme assays of the original serum and depleted serum.

ANALYSIS OF THE EFFECT OF TRANSCRIPTIONAL INDUCERS ON ACHE EXPRESSION: Cells were plated (100,000 cells/well/ml) in 48-well tissue-culture plates using AChE depleted serum medium. Forskolin and dibutyryl-cAMP (Sigma chemical, St Louis, MO) were diluted in AChE depleted medium and various doses of the compounds (.1, .5, 1.0, 1.5, 2.5 for dbcAMP) (1.5, 3, 6, 12, 24, 30, and 60 μM for Forskolin) were added to the cells and incubated for different time periods. Forskolin experiments were also performed by incubating the cells with for a total of 7-10 days. In some cases, a single dose of Forskolin was added at the start of incubation and in other cases Forskolin was added every other day for the incubation period.

ACETYLCHOLINESTERASE (ACHE) MICROASSAY: AChE was determined by the Ellman's method that was adapted for 96-well plates [14;15]. For the AChE assay, an increase in absorbance was monitored at 412 nm for 10 min in a reaction mixture containing 20 μl enzyme, 10 μl of 30 mM acetylthiocholine iodide (ATC), 10 μl of 10 mM dithionitrobenzene, and 50 mM sodium phosphate buffer (pH 8) in a final volume of 300 μl.

Culture supernatant (20 μl) was used as the source of extracellular enzyme. For the estimation of intracellular AChE, the medium was removed and the cells were washed with phosphate buffered saline (PBS) and lysed using ice-cold phosphate buffer containing 1% Nonidet P-40 or with a lysis buffer, T-PER, purchased from Sigma. The homogenate was centrifuged for 10 minutes at 3,000 x g at 4°C and the resulting supernatant was used for the intracellular enzyme assay.

PROTEIN DETERMINATION: Total protein content of the cell lysate was determined by a Coomassie brilliant blue protein-binding assay using bovine serum albumin as standard (Bradford, 1976) (Pierce Chemical Co, IL). Ten microliters of the lysate were added to 300 μl of reaction mixture and the OD was measured at 562 nM in a microplate reader.

KARNOVSKY STAINING OF CELLS FOR THE INDUCTION OF ACHE: The method of AChE cytochemical staining was originally described by Karnovsky and Roots for the demonstration of AChE expression [16]. That Karnovsky method was later modified by Hanker et al and Kobayashi et al [17;18]. Briefly, cells in the 48 well plates were incubated for 15 min in a 1.5 ml sodium phosphate buffer (pH 6), containing 10 mg acetylthiocholine iodide, 0.1 ml of 0.1 M sodium citrate solution, 0.2 ml of 30 mM copper sulphate solution, and 0.2 ml of 5 mM potassium ferricyanide solution at room temperature for 4-8 hours. The cells were washed and observed under an Olympus microscope, model IX51.

DFP TREATMENT AND MTT CYTOTOXICITY ASSAY: Diisopropylfluorophosphate (DFP) (Sigma Chemical) was diluted in AChE depleted medium, added to the cells and incubated for 3 days. Neuronal cell survival was quantified by incubating 2-4 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which yields a blue formazan product in living cells but not in dead cells or their lytic debris [19]. The resulting colored end product was solubilized in 70% isopropanol containing 2% HCl and the absorbance was measured at 620 nm using the microplate reader.

CELL MORPHOLOGY ANALYSIS BY MICROSCOPY: Changes on the cell morphology was analyzed by microscopy using Nikon microscope (20X).

RESULTS

DEPLETION OF SERUM ACHE BY PROCAINAMIDE GEL AFFINITY CHROMATOGRAPHY: To reduce the interference and determine proper expression of AChE following induction with transcriptional enhancers, the serum AChE was depleted using procainamide gel affinity chromatography. A column chromatography was carried out to minimize the time of interaction between serum and procainamide to prevent non-specific binding and elimination of other important serum factors. Acetylcholinesterase present in the serum could skew the results or exhibit feedback inhibition of enzyme expression. Enzyme microassay shows that the AChE was completely depleted from the FBS by this protocol (Fig. 1). Protein estimation results showed a small decrease in the level of protein after depletion indicating that the removal of non specific proteins was minimal following procainamide gel affinity chromatography.

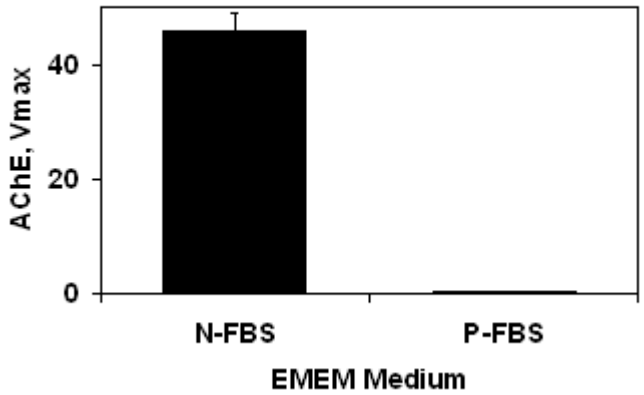

Figure 1. Depletion of serum cholinesterases using procainamide gel affinity chromatography. AChE enzyme assay shows that procainamide gel removes most of the AChE activity present in the serum. Results are expressed as mean ± SEM (n = 4).

EFFECT OF DIBUTYRYLCYCLIC AMP ON THE ACHE EXPRESSION IN NEURO 2A CELL LINE: Neuro 2A cells were (1 X 10^5 cell/well in 48 well plates) incubated with various dilutions (0.1, 0.5, 1, 1.5, and 2.5 nM) of dbcAMP for 7 days. Microscopy results showed that cell morphology of Neuro 2A was altered in dbcAMP-treated cells (Fig 2A). Production of distinct filamentous projections was observed at 0.1 nM, 0.5 nM, 1 nM and 1.5 nM. The neurofilaments started forming networks in 1 and 1.5 dbcAMP-treated cells. It was also found to be cytotoxic to Neuro 2A cells above 1.5 nM.

The extracellular AChE expression in the culture supernatant and the intracellular AChE were measured as described in 'Materials and Methods'. The data shows that extracellular AChE level was gradually increased in dbcAMP-treated cells. The optimum induction of approximately 1.3-fold was observed at 0.5 nM dbcAMP treatment (Fig. 2B). Higher doses were cytotoxic to Neuro 2A cells and consequently the level of AChE was low. The intracellular AChE level was also gradually increased in Neuro 2A cells treated with dbcAMP (Fig. 2C). Consistent with the extracellular AChE level, the highest induction of AChE was observed at 0.5 nM Forskolin treated cells; approximately a 1.5-fold increase from the control. Total cellular protein level was marginally decreased in dbcAMP-treated cells (Fig 2D). The decrease in the total protein may be the result of reduced cell proliferation during cell differentiation and neurite outgrowth dbcAMP at higher doses.

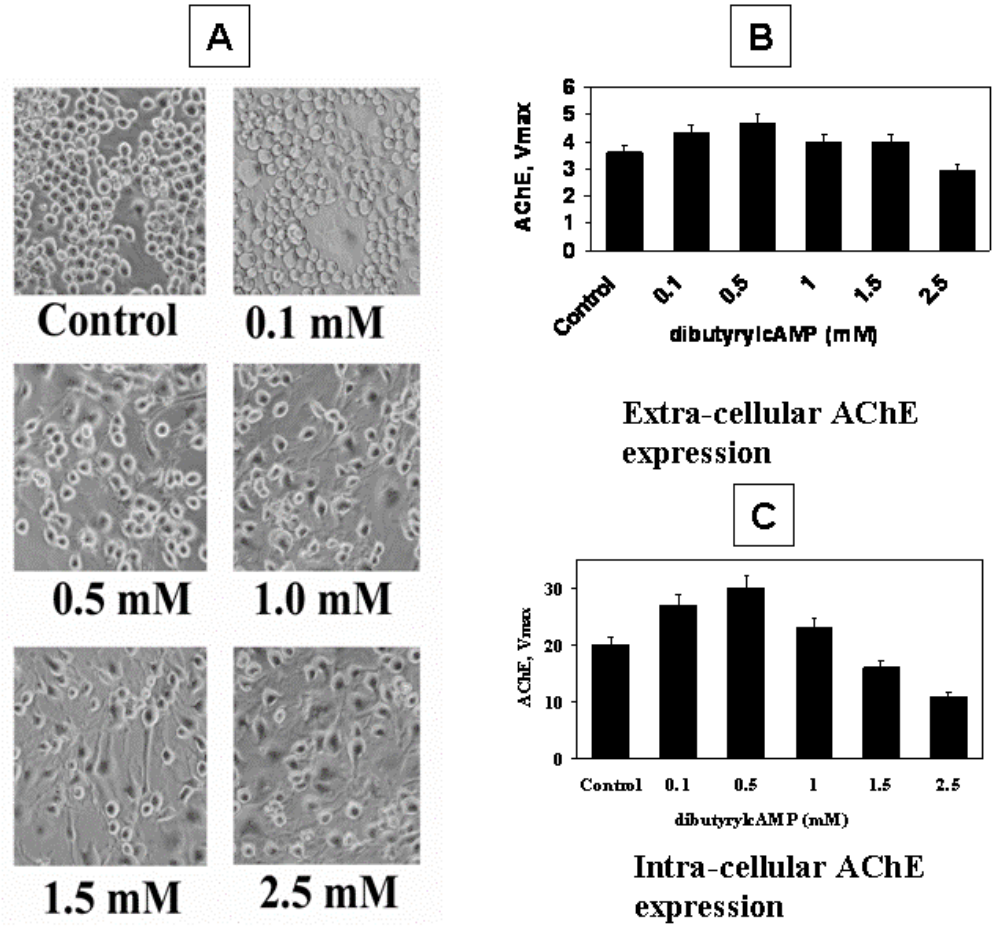

Figure 2. Effect of dibutyryl cAMP on Neuro 2A cells. A. Photomicrographs (20X) of Neuro 2A cells treated without or with various doses of dbcAMP for 7 days. B. Extracellular AChE expression determined by microassay of 20 µl culture supernatant. C. Cells were lysed with 1% nonidet-P40 and the intracellular AChE expression was determined by using 20 µl cell lysate. All the assays were performed in triplicates or quadruplicates. The variations between triplicates were less than 10% of the mean.

FORSKOLIN UPREGULATES ACHE EXPRESSION IN NEURO 2A CELL LINE: Neuro 2A cells were (1 X 10^5 cell/well in 48 well plates) incubated with 2-fold dilutions (1.5, 3, 6, 12, 24, 30, and 60 μM) of Forskolin for 7 days. Microscopy results showed that cell morphology of Neuro 2A was altered in Forskolin-treated cells (Fig 3A). Production of distinct filamentous projections was observed at 6 μM, 12 μM, 24 μM, and 30 μM. The neurofilaments started forming networks in 6 and 12 μM Forskolin treated cells. Forskolin was found to be cytotoxic to Neuro 2A cells above 30 μM.

The extracellular AChE expression in the culture supernatant and the intracellular AChE were measured as described in 'Materials and Methods'. The data shows that extracellular AChE level was gradually increased in Forskolin treated cells. The optimum induction of approximately 2-fold was observed at 30 μM Forskolin treatment (Fig. 3B). Higher doses were cytotoxic to Neuro 2A cells and consequently the level of AChE was low. The intracellular AChE level also increased in Neuro 2A cells treated with Forskolin (Fig. 3C). Contrasting with the extracellular AChE level, highest induction of AChE, a 4-fold induction compared to untreated controls, was observed at 12 μM Forskolin treated cells. Total cellular protein level was marginally decreased in Forskolin treated cells (Fig 3D). The decrease in the total protein may be the result of reduced cell proliferation during cell differentiation and neuronal outgrowth that Forskolin induces at higher doses.

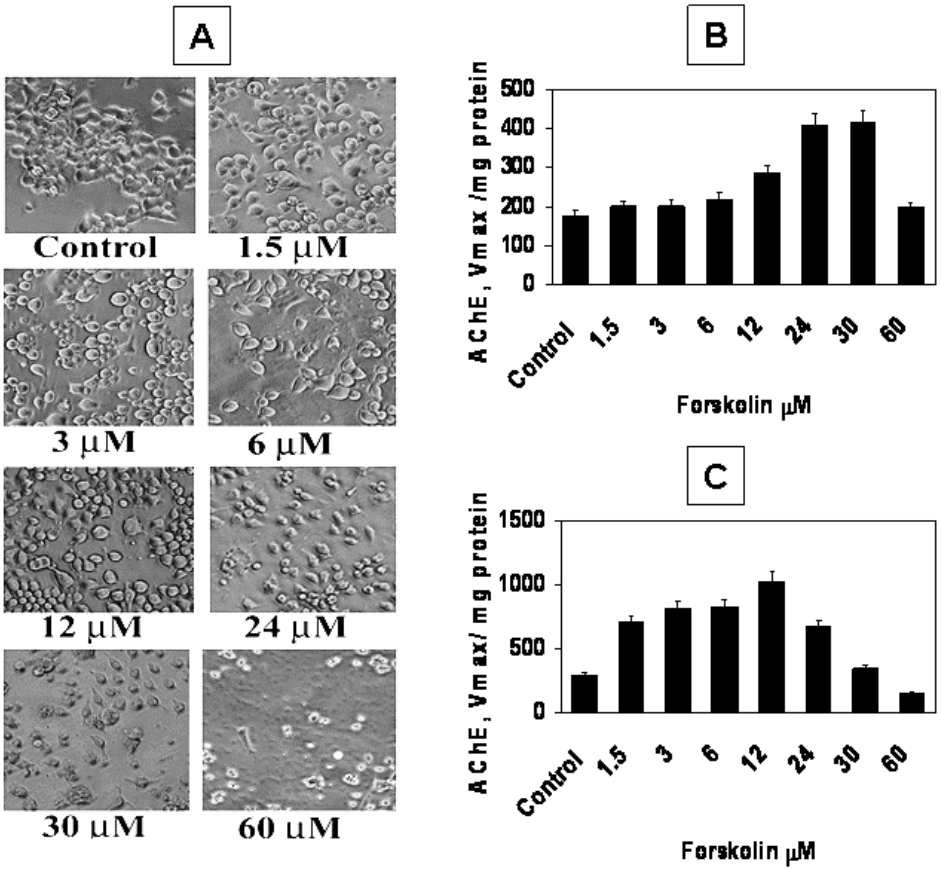

Figure 3. Effect of Forskolin on Neuro 2A cells. A. Neuro 2A cells repeatedly treated with various doses of Forskolin every second day for 10 days. B. Extracellular AChE expression, determined by microassay using 20 μl culture supernatant. C. Intracellular AChE expression. Cells were lysed with 1% nonidet-P40 and the intracellular AChE expression was determined by using 20 μl cell lysate. All the assays were performed in triplicates or quadruplicates and multiple times. The variations between triplicates were less than 10% of the mean.

TREATMENT OF FORSKOLIN EVERY OTHER DAY FOR 7 DAYS INDUCE ACHE EXPRESSION AT LOWER DOSES: To investigate whether continuous addition of Forskolin over an extended period of time is required for optimal induction of AChE, the Forskolin treatment was done every other day for 10 days. For treatment, the medium was completely removed and replaced with fresh medium containing the appropriate amount of Forskolin. Under these conditions, the optimum dose of Forskolin that induced AChE was 24 μM to 30 μM. The expression of extracellular and intracellular AChE in Forskolin treated cells was approximately 2.3 and 2.4-fold higher compared to controls respectively. These results indicate that repeated addition of Forskolin is able to induce AChE expression at lower doses compared to a single addition.

CYTOCHEMICAL STAINING FOR ACHE INDICATE INCREASED EXPRESSION OF THE ENZYME FOLLOWING FORSKOLIN TREATMENT: Neuro 2A cells repeatedly treated with and without Forskolin were stained with Karnovsky staining as described in 'Materials and Methods'. Microscopy imaging showed that the staining was very concentrated in cells treated with 12 nM Forskolin compared to control cells (Fig 3). Strong staining indicates high level expression of AChE. These results further confirm that Forskolin clearly induces the intracellular expression of AChE.

EFFECT OF ORGANOPHOSPHATE DIISOPROPYLFLUOROPHOSPHATE IN NEURO 2A CELLS: To study the protection of Forskolin treatment on the cytotoxic effect of organophosphate exposure, we first analyzed the effect of diisopropylfluorophosphate, a surrogate chemical warfare agent soman and sarin, on Neuro 2A cells. The cells ($1X10^5$ cells/well in 48 well plates) were incubated with 0.24, 0.48, 0.95, 1.9, 3.8 and 7.6 mM DFP for 3 days. Cytotoxic activity of DFP was visible by rounding, floating of dead cells, and reduction in the number of remaining cells. The cell morphology showed that significant cytotoxicity was evident at the lowest dose of 0.24 mM DFP (Fig 4A). MTT cytotoxicity assay results indicated that LD_{50} of DFP on Neuro 2A cells was 1.9 mM (Fig 4B). Thus, although the cytotoxicity was obvious at lower doses of DFP, a significant increase in the dose is required to kills the cells. Extracellular AChE determination showed a strong inhibition in of the AChE at the 0.24 mM DFP that further decreased with an increase in the dose of DFP (Fig 4C).

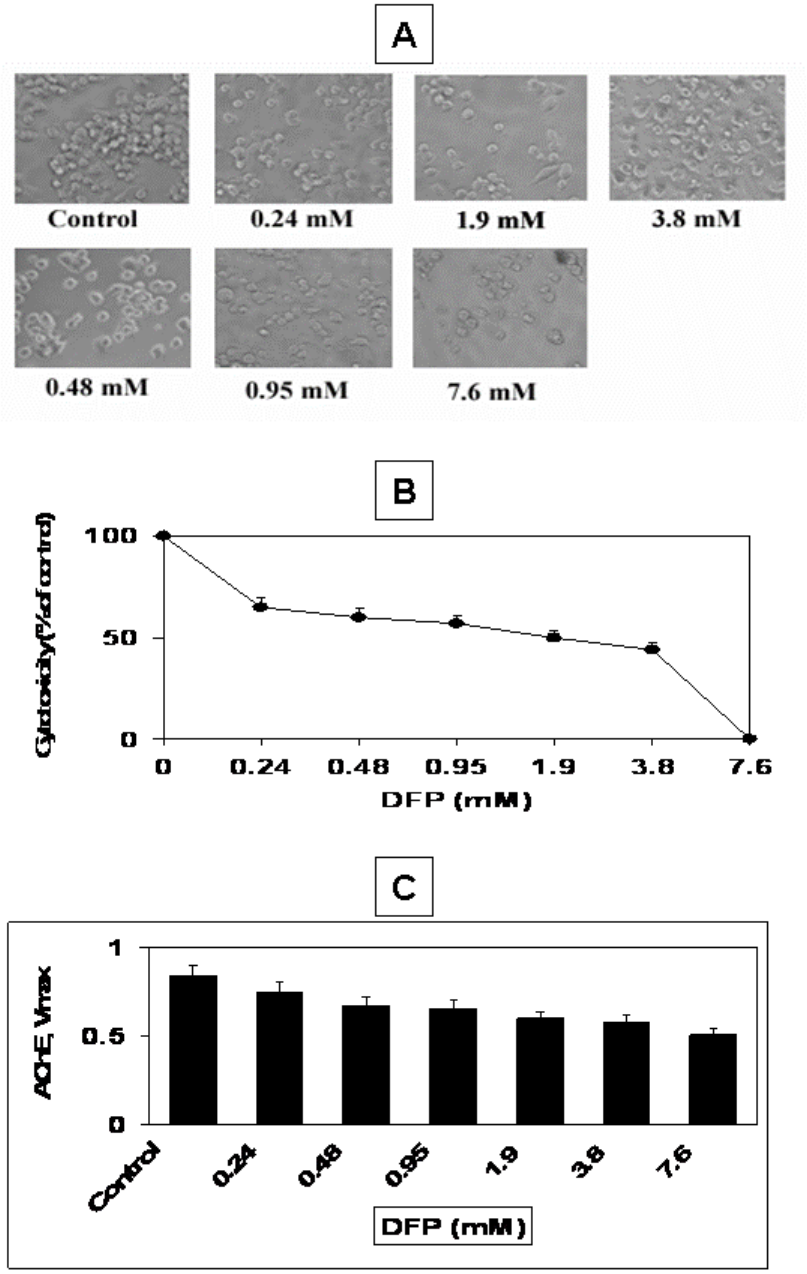

Figure 4. Effect of diisopropylfluorophosphate on Neuro 2A cells. A. Photomicrographs (20X) of Neuro 2A cells treated without or with various doses of DFP for 3 days. B. The cytotoxicity of DFP was measured by MTT assay as described in 'Materials and Methods' and represented as percent of control. C. Extracellular AChE expression was determined by microassay of 20 µl culture supernatant. All the enzyme assays were performed in triplicates. The variations between triplicates were less than 10% of the mean.

FORSKOLIN TREATMENT PROTECTS THE CELLS FORM THE CYTOTOXICITY OF ORGANOPHOSPHATE DIISOPROPYLFLUOROPHOSPHATE: Neuro 2A cells (1 x10^5 cell/well in 48 well plates) were incubated with 1.5, 3, 6, 12, 24, 30, and 60 μM Forskolin for 7 days. DFP (7mM was added and the cells were incubated for 3 days. Microscopy results showed that many of the cells treated with 24 and 30 μM Forskolin followed by DFP survived and retained cellular morphology (Fig 5A). Cell survivability was greatest at 30 μM Forskolin. The protection of Forskolin against DFP cytotoxicity was measured by MTT cytotoxicity assay. Consistent with the cellular morphology, the cytotoxicity data show that 24 and 30 μM Forskolin treatment significantly protected the Neuro 2A cells against DFP exposure (Fig 5B). This collection of data suggests that induction of cellular AChE by Forskolin bio-scavenge the organophosphate and reduces its cytotoxicity and thereby protects against of organophosphate exposure.

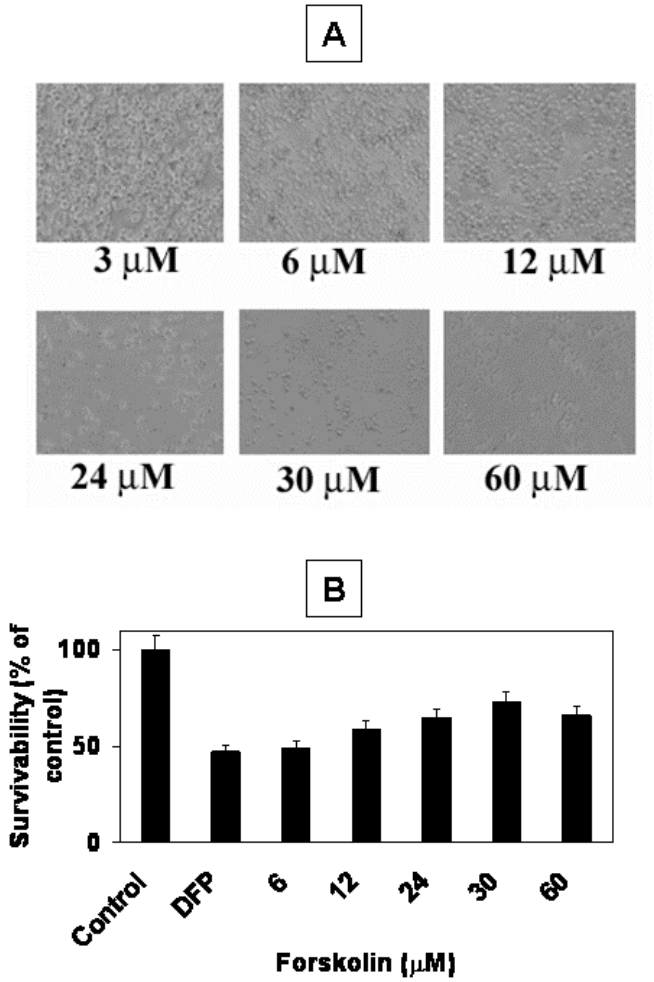

Figure 5. Forskolin protects neuro 2A cells from the cytotoxicity of diisopropylfluorophosphate. A. Photomicrographs (20X) of Neuro 2A cells treated without or with indicated doses of Forskolin and then incubated with 7 mM DFP for 3 days. B. The protective effect of Forskolin was measured in DFP treated cells by MTT cytotoxicity assay as described in 'Materials and Methods'. The protection was expressed as percentage of control cells not treated with DFP and Forskolin. All the assays were performed in triplicates. The variations between triplicates were less than 10% of the mean.

CONCLUSIONS

The major finding in this study is that potent nerve agent bio-scavengers like acetylcholinesterase can be induced in the cells using transcriptional enhancers. The induced AChE was able to protect the cells against the cytotoxicity of a surrogate chemical warfare agent diisopropylfluorophosphate. These results indicate that transcriptional inducers of AChE could be used as novel antidotes for the treatment of chemical warfare agents which are potent inhibitors of cholinesterases. Although drugs based on transcriptional regulation are still being investigated and in the process of development for many disorders that are caused by differential gene expression, our study supports a treatment option for organophosphates and opens new avenues for the treatment of chemical warfare agent exposure.

The approach of using transcriptional inducers for nerve agent exposure has several advantages. Currently, purified fetal bovine serum AChE or human plasma BChE is being used for the treatment of OP exposure. Causing bodily induction of these two enzymes will minimize the production of antibodies formed due to the complex structure and posttranslational modification of cholinesterases. Also the cost of drug, labor, and treatment will be much lower by using transcriptional inducers compared to purified enzyme treatments that need a large amount of human plasma while producing a low yield. Another advantage of using transcriptional inducers is that the enzyme level can be maintained high as long as the inducer is present in the body and will not need invasive methods of administration.

To accurately measure the induced level of AChE expression, we have depleted the fetal bovine serum of AChE/BChE using procainamide gel affinity chromatography. Initial experiments with normal fetal bovine serum showed the presence of very high AChE levels that interfere with the estimation of induced AChE because of the high Vmax value. Procainmide gel completely removed the serum cholinesterases. However, protein estimation showed that few other proteins or factors are removed non-specifically by procainamide gel. Accordingly, cell growth was found be normal in procainamide treated serum compared to untreated serum.

Increased induction of AChE and reduction in the optimal dose following repeated treatment with Forskolin indicate that continuous presence of the drug is required for the induction. This is possibly due to the metabolism and degradation of Forskolin that could result in the reversal of induction. Repeated addition also shows a cumulative effect in the induction of AChE. Experiments are being conducted to evaluate the protection of other organophosphates like MEPQ, DEPQ and amitone following treatment with Forskolin.

In addition to the Neuro 2A cell line, we have done a few experiments with another mouse neuroblastoma P19 cell line and a human neuroblastoma cell line SH-SY5Y. More experiments are needed to prove the potential of AChE expression in mammalian cells by transcriptional inducers. Similarly more experiments with longer incubation with Forskolin and protection by different concentrations of DFP are required to study the exact dose of Forskolin that can protect the highest lethal-dose of DFP or other organophosphate surrogate markers. Although we do not expect Forskolin to induce any other reducing agents containing free SH groups, further study is needed. Alternatively, AChE estimation of the total AChE mRNA by RT-PCR will provide strong evidence of transcriptional induction. In summary, we show that expression of nerve agent bio-scavengers like AChE and BChE can be induced by transcriptional inducers such as Forskolin. The induced enzyme can bio-scavenge the organophosphates, protect the cells from OP induced cytotoxicity, and are potential new ways to treat CWA exposure.

ACKNOWLEDGMENTS

The authors thank the Department of Biochemical Pharmacology/Division of Biochemistry, the SEAP program, and George Washington University for their help in completing this project. Financial support from the Material Research and Medical command of the Department of Defense is greatly acknowledged. Opinions and assertions contained herein are private views of the authors and are not to be construed as official or reflecting the views of the department of the army or the DOD.

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